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(57) Abstract

The present invention relates to a method for conserving cellular adaptive responses to hypoxia in a mammalian host (e.g., human) comprising administering to the host in need thereof an effective amount of hypothalamic inhibitory factor (HIF). The present invention also relates to a method for treating hypoxia in a mammalian host comprising administering to the host in need thereof an effective amount of HIF. In addition, the present invention can be used to treat or prevent hypoxia insult. Also encompassed by the present invention is a method for conserving adenosine triphosphate in a mammalian cell comprising introducing an effective amount of HIF to the cell (e.g., a brain cell, a cardiac cell, a renal cell).

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USE OF HYPOTHALAMIC INHIBITORY FACTOR FOR TREATING HYPOXIA

BACKGROUND

Experimental evidence linking an endogenous digitalis-like sodium-potassium adenosinetriphosphatase (Na^+-K^+-ATPase) inhibitor to fluid and electrolyte homeostasis through regulation of renal sodium excretion and, in the case of dysregulated states, to the pathogenesis of a prevalent human disease, hypertension, stimulated efforts to isolate and characterize such a compound (Blaustein, M.P. and J.M. Hamlyn, *Am. J. Med.* 77:45-59, (1985); Goto, A., *et al.*, *Pharmacol. Rev.* 44:377-399, (1992); Haber, E. and G.T. Haupert, Jr., *Hypertension* 9:315-324, (1987)). Until now, the only known specific regulators of mammalian Na^+-K^+-ATPase were plant-derived digitalis glycosides and bufodienolides found in amphibian species. Recently, a novel compound was isolated from bovine hypothalamus and structurally characterized as an isomer of the plant glycoside, ouabain (Tymiak, A.A., *et al.*, *Proc. Natl. Acad. Sci., USA* 90:8189-8193, (1993)). This hypothalamic inhibitory factor, HIF, had been shown to have biological and biochemical properties similar to, but not identical to, those of ouabain (Haupert, G.T., Jr., In: *The Sodium Pump*. Ed. by B. Bamburg and W. Schoner. New York: Springer Verlag, (1994), p. 732-742). Thus, HIF is a potent inhibitor of the Na pump in renal tubular cells (Cantiello, H.F., *et al.*, *Am. J. Physiol.* 255:F574-F580, (1988)), has positive inotropic activity in cardiac myoctyes (Hallaq, H.A. and G.T. Haupert, Jr., *Proc. Natl. Acad. Sci. USA* 86:10075-10079, (1989)), and vasoconstrictive properties in isolated blood vessels (Janssens, S.P., *et al.*, *J.*

Cardiovasc. Pharmacol. 22:S42-S46, (1993), all consistent with the proposed role for regulation of body fluid volume status and cardiovascular physiology.

Important differences in biological activity from plant ouabain have also been shown. There were differences in binding and dissociation kinetics in the renal cells (Cantiello, H.F., *et al.*, *Am. J. Physiol.* 255:F574-F580, (1988)); inotropic activity in cardiac myocytes occurred at concentrations approximately three orders of magnitude less than for ouabain, with evidence for less toxicity in the cardiac cells (Hallaq, H.A. and G.T. Haupert, Jr., *Proc. Natl. Acad. Sci. USA* 86:10075-10079, (1989); U.S. patent application No. 08/338,264, filed November 10, 1994); and HIF produced potent biological activity in tissues containing isoforms of the Na^+-K^+-ATPase which are highly resistant to ouabain (Haupert, G.T., Jr., In: *The Sodium Pump*. Ed. by B. Bamburg and W. Schoner. New York: Springer Verlag, (1994), p. 732-742).

SUMMARY OF THE INVENTION

The present invention relates to a method for conserving cellular adaptive responses to hypoxia in a mammalian host (e.g., human) comprising administering to the host in need thereof an effective amount of hypothalamic inhibitory factor (HIF).

The present invention also relates to a method for treating hypoxia in a mammalian host comprising administering to the host in need thereof an effective amount of HIF. In addition, the present invention can be used to treat or prevent hypoxia insult.

Also encompassed by the present invention is a method for conserving adenosine triphosphate in a mammalian cell comprising introducing an effective amount of HIF to the cell (e.g., a brain cell, a cardiac cell, a renal cell).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of the study protocol described in the exemplification, wherein rats were kept both in regular cages (21%, O_2, left) and in hypoxic sealed cages (10% O_2, right); brain was removed and half of each midbrain was subsequently incubated *in vitro* at either 95% or 4% O_2; supernatants were collected and new medium rich in NaCl was added to study further release of Na^+-K^+-ATPase inhibitor; the same protocol was applied to adrenal tissue.

Figure 2A is a bar graph of sodium pump inhibition in midbrain tissue slices from CHP20P chromatography under various *in vivo* and *in vitro* oxygen conditions; shaded portion shows area of elution of bovine HIF under identical chromatographic conditions.

Figure 2B is a bar graph of sodium pump inhibition in adrenal tissue slices from CHP20P chromatography under various *in vivo* and *in vitro* oxygen conditions; shaded portion shows area of elution of bovine HIF under identical chromatographic conditions.

Figure 3 is a graph of Na^+-K^+-ATPase inhibition caused by equal doses of purified tissue-slice supernatants in the erythrocyte rubidium uptake (y axis) and purified Na^+-K^+-ATPase inhibition (x axis) assays; linear regression

analysis shows a highly significant correlation between values ($r=0.7$, $p<0.0001$).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based in part on the unexpected discovery that hypoxia is a potent stimulus for the release of hypothalamus inhibitory factor (HIF) and that HIF is involved in energy conserving cellular adaptive responses to hypoxia insult through adenosine triphosphate (ATP) conservation. Thus, the present invention relates to methods for conserving cellular adaptive responses to hypoxia in a mammalian host comprising administering to the mammalian host in need thereof an effective amount of hypothalamic inhibitory factor. The present invention also relates to a method for conserving ATP in a mammalian cell (e.g., brain cell, cardiac cell, renal cell) comprising introducing an effective amount of HIF to the cell. Also encompassed by the present invention is a method for preventing and/or treating adverse consequences of hypoxia (hypoxic insult) in a mammalian host comprising administering to the host in need thereof an effective amount of hypothalamic inhibitory factor.

The mammalian host can be any mammal which is in need of increased cellular or tissular ATP levels in the face of hypoxia, and includes, for example, human, canine, feline, bovine and murine hosts.

An endogenous inhibitor of Na^+-K^+-ATPase has been isolated from bovine hypothalamus and human plasma and structurally characterized as an isomer of the plant cardiac glycoside, ouabain (Tymiak, A.A., *et al.*, *Proc. Natl. Acad. Sci., USA* 90:8189-8193, (1993); and Zhao, N.,

et al., Biochemistry 34:9893-9896, (1995)). This hypothalamic inhibitory factor (HIF) inhibits Na^+-K^+-ATPase with high affinity in cardiovascular and renal tissues consistent with physiologic regulation *in vivo*.

Stimulus(i) for the release of HIF from tissue are unknown. Clinical studies suggest that hypoxia may be a stimulus for the elaboration of digitalis-like activity in humans, and animal studies indicate that high NaCl concentration in the central nervous system stimulates ouabain-like activity. The ability of low O_2 tension *in vivo* and *in vitro* to stimulate HIF release from midbrain and adrenal tissues in Wistar rats was examined. A Na^+-K^+-ATPase inhibitor was recovered from supernatants of these tissues incubated *in vitro*, and this activity co-chromatographed with bovine HIF and showed Na^+-K^+-ATPase inhibitory activity which was identical to the activity of pure HIF in two biological assays. In both tissues, hypoxia stimulated a remarkable release of the inhibitor, and this release was further enhanced by subsequent exposure to 300 mM NaCl. Plasma from rats exposed to hypoxia *in vivo* also showed increased levels of the purified inhibitory activity. As shown herein, hypoxia is a potent stimulus for the release of HIF, and HIF is involved in energy conserving cellular adaptive responses to hypoxic or ischemic insult through ATP conservation.

HIF for use in the present invention can be obtained by purifying HIF from natural sources or chemically synthesizing HIF. In a preferred embodiment, purified HIF is used. Purified HIF refers to HIF which is substantially free of or isolated from other tissue or fluid protein

components and contaminants. Various procedures may be used to purify HIF from natural sources. For example, as described in U.S. patent application No. 08/338,264, filed November 10, 1994, which is incorporated by reference, HIF has been purified to homogeneity using an affinity chromatography method in which purified renal Na^+, K^+-ATPase (e.g., isolated from a canine) is coupled to paramagnetic particles through a glutaraldehyde bridge (Tymiak, A.A., *et al. Proc. Natl. Acad. Sci., USA, 90*:8189-8193 (1993)). The enzyme immobilizes bound HIF in the presence of Mg^{++} and inorganic phosphorous with high affinity, and after washing away contaminating materials, HIF is eluted from the affinity column by chelating Mg^{++} with EDTA. A subsequent HPLC step results in purification of HIF (Tymiak, A.A., *et al. Proc. Natl. Acad. Sci., USA, 90*:8189-8193 (1993)). HIF has also been purified to homogeneity using an immunoaffinity chromatography method in which an antibody which binds to HIF is coupled to the resin of an immunoaffinity column. The antibody recognizing HIF immobilizes bound HIF with high affinity, and after washing away contaminating materials, HIF is eluted from the affinity column. A subsequent HPLC step can be used to further purify HIF. See U.S. patent application No. _____, filed May 30, 1997, Attorney Docket No. BION97-01, which is incorporated by reference. It should be noted that pharmaceutically acceptable salts of HIF are also contemplated. Suitable salts include those well known to those of skill in the art.

As defined herein, an "effective amount" is an amount sufficient, when administered to the host, to result in a

conserved cellular adaptive response to hypoxia in the mammalian host relative to the cellular adaptive response to hypoxia when an effective amount of HIF is not administered. An "effective amount" of HIF as defined herein also refers to the amount of HIF which upon introduction into a mammalian tissue results in the conservation of ATP. Additionally, an "effective amount" also refers to an amount sufficient to ameliorate the effects of hypoxia. The amount of HIF used to treat a host will vary depending on a variety of factors, including the size, age, body weight, general health, sex and diet of the host. In addition, an effective amount of HIF will depend on the nature of the disease being treated, and can be determined by standard clinical techniques. The precise dose to be employed will also depend on the route of administration and the seriousness of the disease or disorder, and should be decided according to the judgement of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous or intraarterial administration are generally about 4.5 ug to about 20 ug per kilogram body weight. In one embodiment the dosage is about 4.5 ug per kilogram body weight, in another embodiment the dosage is about 12 ug per kilogram body weight, and in a further embodiment the dosage is about 20 ug per kilogram body weight. Suitable dose ranges for oral administration of HIF are the same as for intravenous administration of HIF, but the HIF dosage is administered orally over several days. Effective doses may be extrapolated from dose response curves derived from *in vitro* or animal test models.

The HIF of the present invention can also be administered prophylactically to a host as a method of preventing the conditions described herein. Alternatively, HIF can be administered therapeutically to a host as a method of treating an existing disease and/or condition in the host, and can result in amelioration or elimination of the disease and/or condition.

The formulation and route of delivery of HIF to the host can be accomplished in a variety of ways. Routes of administration include intradermal, transdermal (e.g. slow release polymers), intramuscular, intraperitoneal, intraarterial, intracardiac, intravenous, subcutaneous, oral, epidural, and intranasal routes. Any other convenient route of administration can be used, for example, infusion or bolus injection, or absorption through epithelial or mucocutaneous linings. The HIF can be administered together with other components or biologically active agents, such as pharmaceutically acceptable surfactants (e.g., glycerides), carriers (e.g., saline, buffered saline, dextrose, water, glycerol, ethanol), excipients (e.g., lactose), diluents and vehicles and combinations thereof. The formulation should suit the mode of administration.

The composition can also include, if desired, minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of

mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In a preferred embodiment, the HIF is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous or intraarterial administration to the mammalian host (e.g., a human). For example, HIF for intravenous administration can be a solution in sterile isotonic aqueous buffer. Where necessary, the HIF composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampule or sachette indicating the quantity of active agent. Where the composition is administered by injection, an ampule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration. Thus, the invention also relates to the use of HIF in the manufacture of medicaments for the treatment or prevention of hypoxia, hypoxic insult and/or conserving ATP in a mammalian cell or tissue.

As described herein, the effect of hypoxia on the release of endogenous Na^+-K^+-ATPase inhibitory activity from rat brain and adrenal tissues was examined and a remarkable increase in release from both tissues of an inhibitor which coelutes with purified bovine brain HIF, in response to hypoxic challenge was shown. Thus, the invention can be used to conserve cellular adaptive responses to hypoxia in a mammalian host. In particular,

the invention can be used to prevent and/or treat a patient with hypoxia.

Crabos and co-workers appear to have demonstrated that a Na^+-K^+-ATPase inhibitor was released by rat midbrain slices *in vitro* and that this release was inhibited by atrial natriuretic peptide (Crabos, M., *et al.*, *Am. J. Physiol.* 254:F912-F917, (1988)). Some recent clinical studies have raised the possibility that the appearance of digitalis-like activity in human serum may be related to low oxygen tension. Thus, increased levels of a digoxin-like immunoreactive factor and/or Na^+-K^+-ATPase inhibitory activity have been reported in the plasma of normal individuals at high altitude (DeAngelis, C., *et al.*, *Am. J. Hypertens.* 5:600-607, (1992)), in patients with chronic respiratory failure (Ferri, C., *et al.*, *Clin. Sci.* 87:447-451, (1994); and Varsano, S., *et al.*, *Chest* 101:146-149, (1992)) and in normal subjects undergoing voluntary hypoventilation (Bagrov, A.Y., *et al.*, *Hypertension* 26:781-788, (1995)). Furthermore, an altered response to O_2 administration in chronically hypoxic patients has been reported (DeAngelis, C., *et al.*, *Am. J. Nephr.* 13(3):173-177, (1993)). In reviewing older literature, Rein, before formulation of the concept of an endogenous Na^+-K^+-ATPase inhibitor, reported the presence of a "strophanthin-like" substance in blood of animals exposed to hypoxia (Rein, H., *Naturewissen-schaften* 36:233-239: 260-268, (1949)).

As shown herein, the release of an endogenous Na^+-K^+-ATPase inhibitor in response to hypoxia at the cellular level can be used to save energy adaptive responses in

certain tissues. Specifically, down-regulation of ion-channel activity in turtle brain, with resulting lower demand for depleted ATP stores, has been postulated to account in part for this species' remarkable resistance to anoxic brain injury (Lutz, P.L., *Ann. Rev. Physiol.* 54:601-618, (1992)). Since in many metabolically active cell types the Na^+-K^+-ATPase is the major consumer of cellular energy produced by mitochondria as ATP, its inhibition in response to hypoxic insult contributes to such energy-saving cellular adaptive responses.

This invention is illustrated further by the following exemplification, which is not to be construed as limiting in any way.

EXEMPLIFICATION

METHODS

Animals

Two groups of Wistar rats (250-350 g) were kept for three days in different experimental conditions. The first group was housed in a regular cage at room temperature; the second, in a special sealed cage under an artificial air, poor in oxygen (10%). The air composition in the latter was checked every day and the range of oxygen levels was between 8.8 and 10.3% (CO_2 range: 0.09-0.19% and N_2 range: 88.6-90.1%). Both groups of rats were maintained on the same diet (Rodent Laboratory Chow #5001, Purina Mills, Inc.) and allowed free access to water.

Tissue incubations

Rats were anaesthetized using sodium pentobarbital (6.5 mg/100g). Blood samples were collected from each rat in tubes containing sodium heparin, and adrenals and brain were removed and washed twice in a chilled buffer solution (HEPES 10mM, NaCl 114mM, KCl 5mM, $MgSO_4$ 1.15mM, d-glucose 10mM, $NaHCO_3$ 25mM, $CaCl_2$ 2.5mM, NaH_2PO_4 1mM, pH 7.4).

Brains were divided into two halves, cortex was removed and the midbrain dissected free. Slices of midbrain tissue of each half (average weight 22.7 mg) were placed in a culture plate insert (Millicell CM, Millipore) and incubated (37°C) in the HEPES-buffer medium (1250µl) for 3 h in two different incubators, one with a gas mixture of 5% CO_2-95% O_2, the second with 5% CO_2-4% O_2-91% N_2 (Fig. 1). After 3 h of incubation, supernatants were collected and fresh medium (1180 µl) plus 70 µl of 3M NaCl (final concentration 300 Mm) added. After 1 h of incubation in these conditions the supernatants were collected. All supernatants were acidified with 10 µL of 12N acetic acid and heated to boiling to precipitate proteins. Samples were centrifuged at 16000 g for 15 min., the supernatants collected and neutralized with 5N NaOH to pH 7.4. The same procedure was followed for slices from adrenals. Peripheral blood was collected and centrifuged at 2000 g for 15 min, the plasma separated, acidified, boiled, centrifuged and neutralized to pH 7.4, as for the other samples.

In all there were 15 samples coming from each of 8 different conditions of midbrain incubations, and 3 samples from each of 8 adrenal incubations. The experimental conditions can be summarized as follows (see Fig. 1 for schematic): Group 1: 15 supernatants from midbrain tissue

of rats kept in regular cages with 21% O_2 and subsequently incubated *in vitro* with 95% O_2; group 2: 15 supernatants from midbrain tissue of rats kept in regular cages with 21% O_2 and incubated with 4% O_2; group 3: 15 supernatants from midbrain tissue of rats kept in special cages with 10% O_2 and incubated with 95% O_2; group 4: 15 supernatants from midbrain tissue of rats kept in special cages with 10% O_2 and incubated with 4% O_2; groups 5-8: 15 supernatants from midbrain tissue treated respectively as groups 1-4, but after the incubation with 300 Mm NaCl; group A1: 3 supernatants from adrenal tissue of rats kept in regular cages with 21% O_2 and incubated with 95% O_2; group A2: 3 supernatants from adrenal tissue of rats kept in regular cages with 21% O_2 and incubated with 4% O_2; group A3: 3 supernatants from adrenal tissue of rats kept in special cages with 10% O_2 and incubated with 95% O_2; group A4; 3 supernatants from adrenal tissue of rats kept in special cages with 10% O_2 and incubated with 4% O_2; Groups A5-A8: 3 supernatants from adrenal tissue corresponding to above conditions A1-A4, but following the subsequent incubation with 300 Mm NaCl.

To purify and concentrate activity and determine retention time of inhibitory activity in comparison with HIF derived from bovine hypothalamus, supernatants from each tissue group and respective incubation condition were combined and chromatographed using CHP-20P resin in a reverse phase technique. Plasma samples were chromatographed in the same manner.

CHP-20P chromatography

The method was a cornerstone in the complete purification of HIF from bovine hypothalamus as previously described (Tymiak, A.A., *et al.*, *Proc. Natl. Acad. Sci., USA* 90:8189-8193, (1993)). Briefly, 30 ml of CHP20P resin (Mitsubishi) was activated by using 10 column volumes of HPLC grade methanol followed by 15 column volumes of water. The aqueous sample was loaded and the column eluted at a flow rate of 2 ml/min for 120 min developed as a linear gradient of 0-100% HPLC grade methanol. Thirty 8ml fractions were collected and aliquots from each fraction were dried, reconstituted in buffer and assayed for Na^+-K^+-ATPase inhibitory activity as $^{86}Rb+$ uptake into human erythrocytes by a slight modification of the method previously described (Carilli, C.T., *et al.*, *J. Biol. Chem.* 260:1027-1030, (1985)).

^{86}Rubidium uptake assay

Sodium pump activity was estimated as ouabain-sensitive $^{86}Rb+$ uptake into human red blood cells from healthy donors. Briefly, erythrocytes were washed and suspended to 50% cells in a HEPES buffer pH 7.4 (HEPES 20 mM, $CaCl_2$ 1 mM, $MgSO_4$ 1 mM, NaH_2PO_4 5 mM, NaCl 138 mM, Glucose 11 mM). One ml aliquots of each chromatographic fraction were dried and reconstituted with 50 μl of buffer and incubated at 37°C with 50 μl of erythrocyte suspension and 4 μCi/ml $^{86}RbCl$ for 90 min. The reaction was quenched with 750 μl of cold HEPES buffer (4°C). Unbound counts were separated from cells by spinning through a cushion of silicon/phthalate oil (1:1) and erythrocyte $^{86}Rb+$ determined by gamma emission.

Active fractions from the CHP-20P chromatography were pooled, dried and reconstituted with 1 ml of methanol. Samples of 50, 100 and 200 µl in duplicate were taken and assayed again for $^{86}Rb+$ uptake and for inhibition of purified Na^+-K^+-ATPase in a coupled enzyme assay as previously described (Haupert, G.T., Jr., *et al.*, *Am. J. Physiol. 247 (Renal Fluid Electrolyte Physiol 16)*:F919-F924, (1984)).

An additional experiment was performed to address the question of whether or not effects of NaCl addition could be accounted for by the presence of the salt rather than increased osmolality. Midbrain and adrenal slices were incubated under 5% CO2-95%O2 in the standard HEPES buffer, in buffer supplemented with NaCl (300 mM), and buffer supplemented with choline chloride (final concentration, 300 mM). Supernatants of these incubations were treated, purified, and assayed for sodium pump inhibitory activity as described.

Statistical analysis

The combined supernatants for each condition from slices of each half tissue, midbrain and adrenals, were compared using the paired Student's t test. A p value $\leq$ 0.05 was considered significant.

RESULTS

Chromatographic concentration of Na^+-K^+-ATPase inhibitory activity

Figures 2A-2B shows the chromatographic profiles for Na^+ pump inhibitory activity from midbrain (Fig. 2A) and

adrenal (Fig. 2B) supernatants under the various oxygenation conditions. Inhibitory activity under all circumstances chromatographed with a retention time from seventy-six to ninety-six minutes (10 collected fractions, flow rate 2ml/min). This retention corresponded exactly with that found for bovine HIF (shaded region) suggesting but not proving that the inhibitor recovered from brain and adrenal supernatants is the same as the bovine hypothalamic inhibitor structurally characterized as an isomer of ouabain (Tymiak, A.A., *et al., Proc. Natl. Acad. Sci., USA* 90:8189-8193, (1993)).

To further characterize the biological activity recovered from this chromatography, aliquots of the pooled active fractions were tested for specific inhibition of pure Na^+-K^+-ATPase prepared from dog renal outer medulla by the method of Jorgensen (Jorgensen, P.L., *Biochim. Biophys. Acta* 356:36-52, (1974)). This serves as a confirmatory assay used previously to characterize kinetic aspects of the interaction HIF with the pure enzyme. Parallel to earlier findings with bovine HIF (Haupert, G.T., Jr., *et al., Am. J. Physiol. 247 (Renal Fluid Electrolyte Physiol 16)*:F919-F924, (1984)), purified midbrain supernatants produced dose-related inhibition in the coupled enzyme inhibition assay and this correlated well with the active transport inhibition assay in human erythrocytes ($r=0.7$, $p<0.0001$, Fig. 3).

Effects of oxygen tension and NaCl on tissular release of Na^+,K^+-ATPase inhibitory activity

There was an increase in inhibitory activity in supernatants from midbrain and adrenal tissue samples incubated *in vitro* under 4% O_2 in all examined conditions when compared to paired tissues exposed to high O_2 tensions *in vitro* (Table 1, groups 1-4; groups A1-A4). Subsequent exposure of both tissues to high NaCl concentration stimulated further release of inhibitor from midbrain (Table 1, groups 5-8) and adrenal slices (groups A5-A8), and this was accentuated by *in vitro* hypoxia except in midbrain tissues from animals exposed to chronic hypoxia in the *in vivo* phase of the experiment (groups 7 v. 8). Since equimolar addition of choline chloride did not produce any increase in endogenous inhibitor release (see Methods), the effect of NaCl seems to be due to a specific stimulus and not to change in osmotic strength of the bathing medium. This finding is consistent with results of intrathecal administration of NaCl *in vivo* (Huang, B.S., *et al., Circ. Res.* 71:1059-1066, (1992)).

In adrenal tissue, serial exposure *in vivo* then *in vitro* to abundant oxygen revealed comparatively modest baseline release of inhibitory activity (Group A1), while in group A2, where *in vitro* hypoxia was a very effective stimulant, subsequent NaCl addition failed to produce further inhibitor release, suggesting depletion of stores of the inhibitor in adrenal tissue already stimulated to release it by *in vitro* hypoxia (group A2 v. group A6).

Chronic *in vivo* exposure to hypoxia also produced enhanced Na^+,K^+-ATPase inhibitor as measured in blood. Extracted plasma from rats living in the rarified oxygen

environment caused a decrease in human erythrocyte sodium pump activity which was significantly greater than that produced by blood from rats living at atmospheric oxygen conditions (1.366±0.38 v. 0.685±0.29 pmol $^{86}Rb+$ uptake/h/g, respectively $p<0.02$). These results in blood were echoed by adrenal tissue studies where chronic *in vivo* exposure to hypoxia was associated with a large release of inhibitory activity from that tissue on subsequent *in vitro* exposure to high oxygen tension, compared with adrenal tissue from rats which had experienced atmospheric oxygen levels *in vivo* (group A3 v. A1). These results suggest that adrenals produce modest amounts of the Na^+-K^+-ATPase inhibitor under basal conditions (group A1), but that *in vivo* hypoxia activates synthesis of the inhibitor which is then available for release by subsequent stimulus.

The results of tissues exposed to different oxygen tensions *in vivo* but to the same (subsequent) *in vitro* oxygen concentrations were compared (groups 1 v. 3, 2 v. 4; A1 v. A3, A2 v. A4), which showed that midbrain and adrenal tissues responded differently. In midbrain, chronic *in vivo* exposure to room air was associated subsequently with a greater release of inhibitor after *in vitro* incubation at both 95% and 4% oxygen (group 1 v. 3, $p<0.02$; group 2 v. 4, $p<0.0003$). But, as indicated just above, adrenal tissue exposed *in vivo* to atmospheric conditions with subsequent *in vitro* incubation under high oxygen produced modest release of inhibitor when compared to adrenal tissue which had seen initial chronic *in vivo* hypoxia (group A1 v. A3). Also different from midbrain, adrenal tissue ultimately

exposed to low *in vitro* oxygen concentrations appeared indifferent to prior *in vivo* air oxygen content since under both *in vivo* exposures, low *in vitro* oxygen tension stimulated important and nearly identical inhibitor release (group A2 v. A4).

These results suggest that in midbrain tissue accumulation of the inhibitor is relatively constant with *in vivo* normoxia improving the response to subsequent *in vitro* stimulus, while *in vivo* hypoxia attenuates midbrain tissue *in vitro* unstimulated (95% O2) and stimulated (4% O2) release. On the other hand, in adrenal tissue the basal release of inhibitor is relatively low under normoxic conditions, but increases dramatically with hypoxic stimulus whether the latter be *in vivo* or *in vitro*.

Sodium chloride is a potent stimulus to release of the Na^+-K^+-ATPase inhibitor in both midbrain and adrenal tissues. The single exception to this occurred in adrenal tissue unstimulated *in vivo* (atmospheric O_2), but stimulated *in vitro* (4% O_2, group A2). In this case, subsequent exposure to high NaCl concentration (group A6) produced minimal additional release of the inhibitor, as if stores in these cells had been previously depleted by the prior stimulus of *in vitro* hypoxia.

DISCUSSION

In summary, hypoxia is a potent stimulus for the release of HIF from both midbrain and adrenal tissues *in vitro*, and this release is in general further stimulated by high NaCl concentration. The substance co-chromatographs

with and has Na^+-K^+-ATPase inhibitory activity in two bioassays parallel to HIF, a mammalian isomer of ouabain with demonstrated regulation of Na^+ pump activity in cardiovascular and renal cells. Without being limited by any theory, the present invention is based, in part, on the finding that the mammalian response to hypoxia is release of HIF. Thus, the invention encompasses the administration of an effective amount of HIF in a method for treating hypoxia in a mammalian host.

In Table 1, the characteristics of each group are specified, i.e. 21-95% O_2 describes rats kept at 21% O_2 *in vivo* and tissue slices incubated at 95% O_2 *in vitro*. Sodium chloride (final concentration 300 mM) was added into the media of the groups labeled $+Na^+$. The p values show differences between groups as calculated by Student's t test for paired samples, n = 15 supernatants for each condition (midbrain; n = 3 supernatants for each condition (adrenal) (see Methods). Amounts of the purified inhibitor are expressed in the bioassay as relative decrease in ouabain-sensitive Rb^+ uptake (cellular Na^+ pump activity) per hour incubation, normalized for grams of tissue incubated. Baseline mean Rb^+ uptake was 60 nmol $Rb^+/h/10^6$ cells (n=7 healthy donors).

Table 1

Levels of Na^+ pump inhibitory activity in purified supernatants of midbrain and adrenal slices, as measured by decrease in ouabain-sensitive $^{86}RB^+$ uptake in human erythrocytes (pmol K^+/h/g).

GROUP (midbrain)	50µl	100 µl	200 µl	p VALUES
1. (21-95% O_2)	0.48 ± 0.11	0.71 ± 0.09	1.50 ± 0.01	<0.0001
2. (21-4% O_2)	3.46 ± 0.27	3.38 ± 0.02	4.53 ± 0.09	
3. (10-95% O_2)	0.30 ± 0.09	0.46 ± 0.03	0.92 ± 0.26	<0.0003
4. (10-4% O_2)	0.97 ± 0.04	0.88 ± 0.13	1.53 ± 0.08	
5. (21-95% O_2+Na^+)	1.88 ± 0.38	2.23 ± 0.49	4.34 ± 0.60	<0.0001
6. (21-4% O_2+Na^+)	9.60 ± 0.69	10.36 ± 0.65	12.76 ± 1.77	
7. (10-95% O_2+Na^+)	3.25 ± 0.24	4.55 ± 1.44	4.93 ± 0.55	n.s.
8. (10-4% O_2+Na^+)	3.18 ± 0.36	3.95 ± 0.95	5.76 ± 0.42	

GROUP (adrenal)	50 µl	100 µl	200 µl	p VALUES
A1. (21-95% O_2)	0	0.16 ± 1.83	1.67 ± 0.22	<0.0001
A2. (21-4% O_2)	27.48 ± 0.53	27.87 ± 1.01	29.89 ± 1.00	

A3. (10-95% O_2)	19.25 ± 2.19	16.25 ± 6.24	20.82 ± 1.31	<0.01
A4. (10-4% O_2)	22.17 ± 1.94	26.38 ± 1.18	29.54 ± 0.46	
A5. (21-95% O_2+Na^+)	45.81 ± 7.42	45.39 ± 2.12	68.25 ± 1.05	<0.0006
A6. (21-4% O_2+Na^+)	9.13 ± 2.46	10.78 ± 4.81	7.00 ± 2.95	
A7. (10-95% O_2+Na^+)	27.49 ± 10.93	32.64 ± 2.97	66.97 ± 7.26	<0.008
A8. (10-4% O_2+Na^+)	22.16 ± 7.54	17.55 ± 0.37	47.15 ± 1.16	

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

I claim:

1. A method for conserving cellular adaptive responses to hypoxia in a mammalian host comprising administering to the host in need thereof an effective amount of hypothalamic inhibitory factor.

2. The method of Claim 1 wherein the mammalian host is a human.

3. The method of Claim 1 wherein the hypothalamic inhibitory factor is administered intravenously.

4. A method for treating hypoxic insult in a mammalian host comprising administering to the host in need thereof an effective amount of hypothalamic inhibitory factor.

5. The method of Claim 4 wherein the mammalian host is a human.

6. The method of Claim 4 wherein the hypothalamic inhibitory factor is administered intravenously.

7. A method for conserving adenosine triphosphate in a cell comprising introducing an effective amount of hypothalamic inhibitory factor to the cell.

8. The method of Claim 7 wherein the cell is selected from the group consisting of: a brain cell, a cardiac cell and a renal cell.

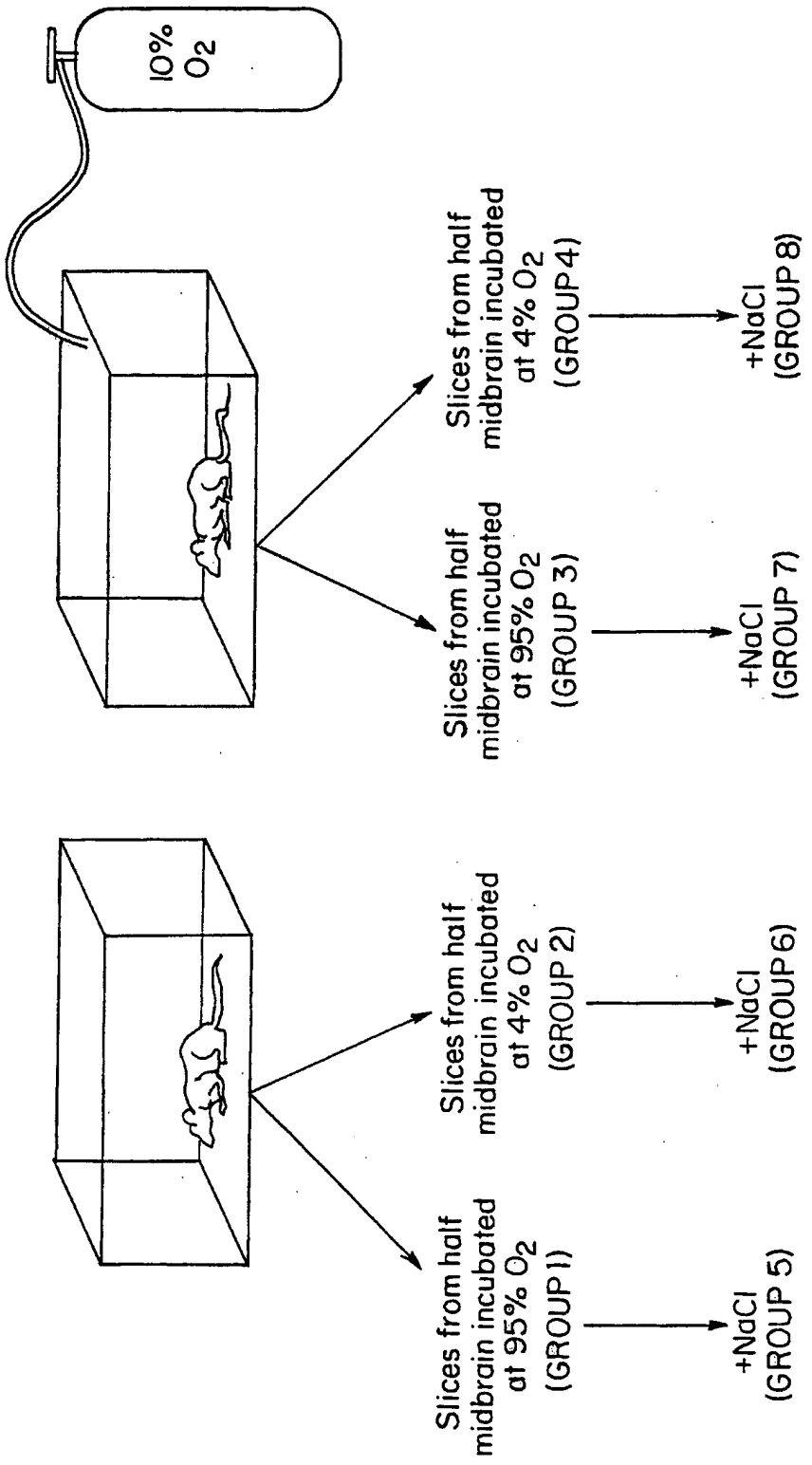
10% O_2
Slices from half midbrain incubated at 95% O_2 (GROUP 1)
+NaCl (GROUP 5)
Slices from half midbrain incubated at 4% O_2 (GROUP 2)
+NaCl (GROUP 6)
Slices from half midbrain incubated at 95% O_2 (GROUP 3)
+NaCl (GROUP 7)
Slices from half midbrain incubated at 4% O_2 (GROUP 4)
+NaCl (GROUP 8)

FIG. 1

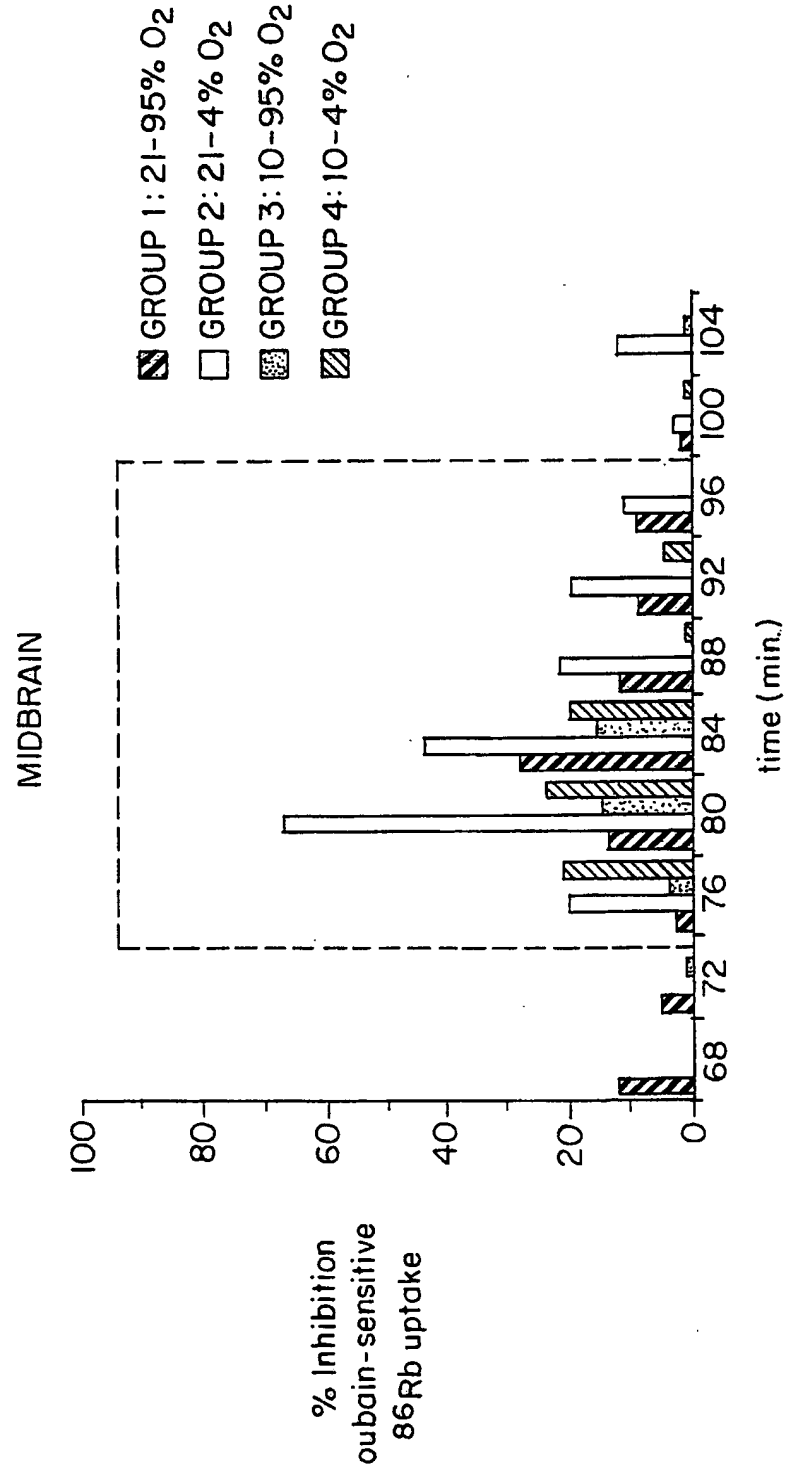
GROUP 1: 21-95% O_2
GROUP 2: 21-4% O_2
GROUP 3: 10-95% O_2
GROUP 4: 10-4% O_2
MIDBRAIN
% Inhibition oubain-sensitive ^{86}Rb uptake
0
20
40
60
80
100
68
72
76
80
84
88
92
96
100
104
time (min.)

FIG. 2A

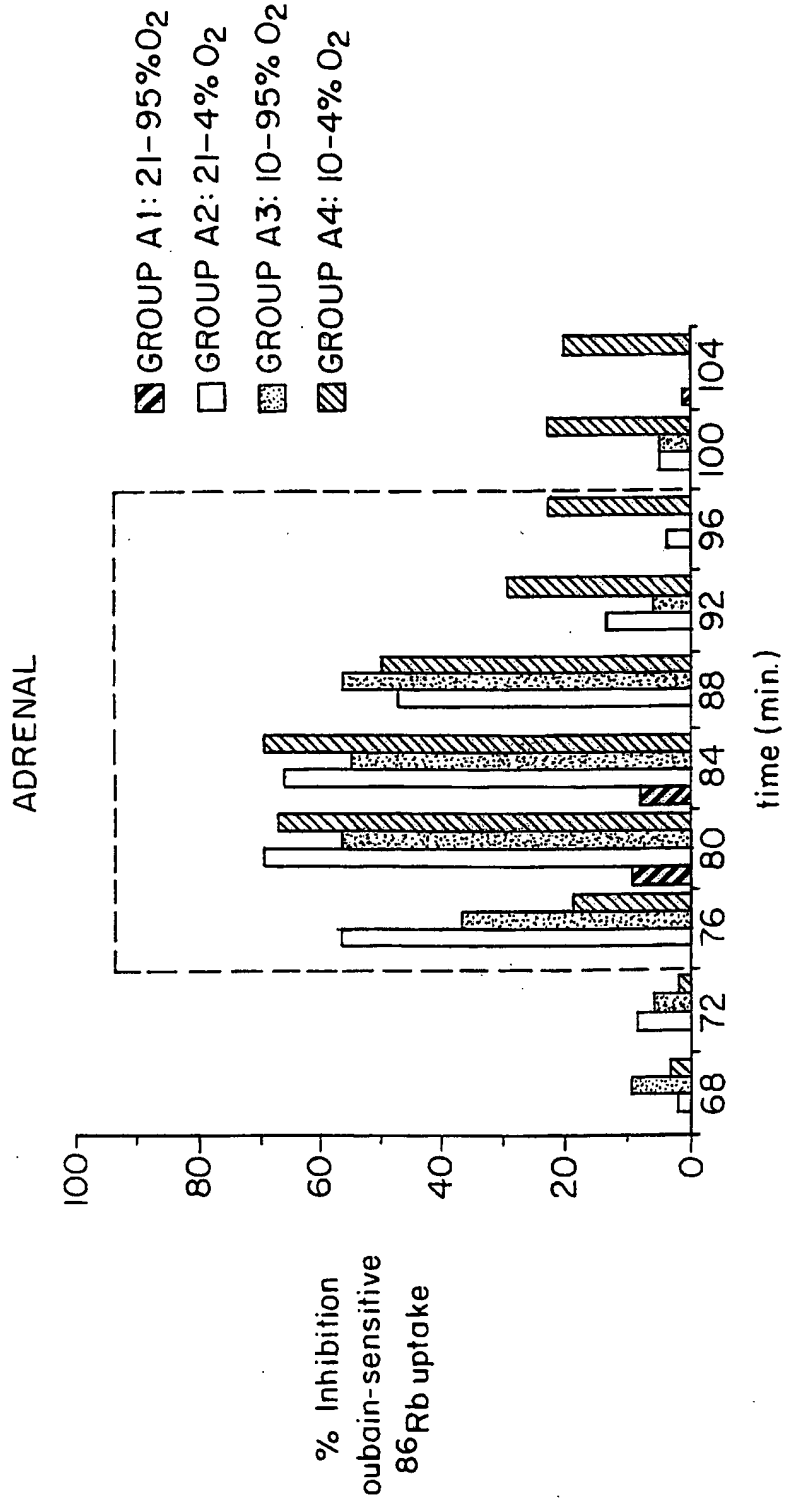
GROUP A1: 21-95% O2
GROUP A2: 21-4% O2
GROUP A3: 10-95% O2
GROUP A4: 10-4% O2
ADRENAL
% Inhibition oubain-sensitive 86Rb uptake
time (min.)
0
20
40
60
80
100
68
72
76
80
84
88
92
96
100
104

FIG. 2B

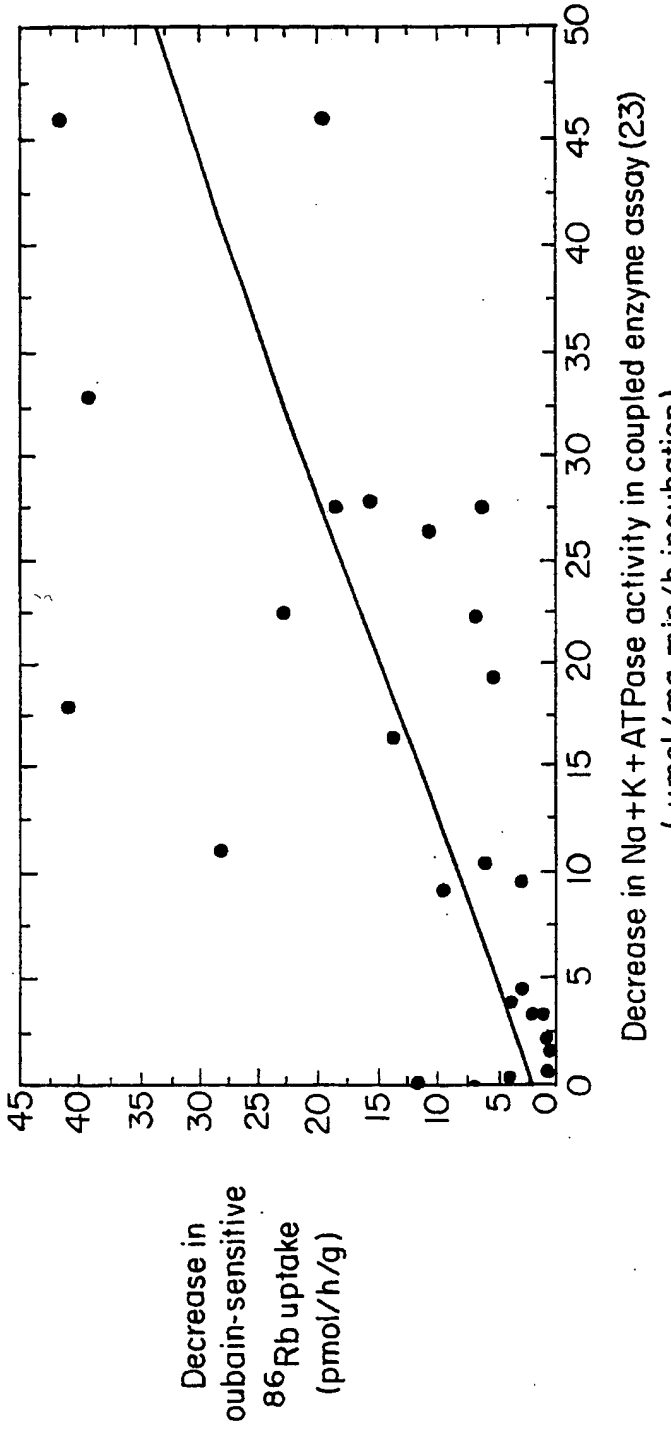
Decrease in ouabain-sensitive ^{86}Rb uptake (pmol/h/g)
0
5
10
15
20
25
30
35
40
45
Decrease in Na+K+ATPase activity in coupled enzyme assay (23) (μmol/mg.min/h incubation)
0
5
10
15
20
25
30
35
40
45
50

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10889

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/705

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	C. DE ANGELIS ET AL.: "HYPOXIA TRIGGERS RELEASE OF AN ENDOGENOUS INHIBITOR OF NA+-K+-ATPase FROM MIDBRAIN AND ADRENAL" AMERICAN JOURNAL OF PHYSIOLOGY, vol. 43, no. 1, January 1998, pages F182-F188, XP002081417 see the whole document ---	1-8
X	C. DE ANGELIS ET AL.: "HYPOXIA PROMOTES IN VITRO RELEASE OF THE HYPOTHALAMIC NA+/K+- ATPase (NKA) INHIBITOR FROM RAT MIDBRAIN" J. AM. SOC. NEPHROL., vol. 4, no. 3, 1993, page 436 XP002081418 see abstract no. 19P --- -/--	1-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 October 1998	02/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hoff, P

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10889

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
X	C. DE ANGELIS ET AL.: "EFFECTS OF HIGH ALTITUDE EXPOSURE ON PLASMA AND URINARY DIGOXIN-LIKE IMMUNOREACTIVE SUBSTANCE" AMERICAN JOURNAL OF HYPERTENSION, vol. 5, no. 9, 1992, pages 600-607, XP002081419 cited in the application see the whole document ---	1-8
A	N. ZHAO ET AL.: "NA,K-ATPase INHIBITORS FROM BOVINE HYPOTHALAMUS AND HUMAN PLASMA ARE DIFFERENT FROM OUABAIN: NANOGRAM SCALE CD STRUCTURAL ANALYSIS" BIOCHEMISTRY, vol. 34, 1995, pages 9893-9896, XP002081420 cited in the application see the whole document ---	1-8
X	EP 0 433 074 A (GEN HOSPITAL CORP) 19 June 1991 cited in the application see the whole document ---	1-8
A	S. VARSANO ET AL.: "ENDOGENOUS DIGOXIN-LIKE IMMUNOREACTIVE FACTOR IS ELEVATED IN ADVANCED CHRONIC RESPIRATORY FAILURE" CHEST, vol. 101, no. 1, 1992, pages 146-149, XP002081421 cited in the application see the whole document ---	1-8
A	C. FERRI ET AL.: "PLASMA ENDOGENOUS DIGOXIN-LIKE SUBSTANCE LEVELS ARE DEPENDENT ON BLOOD O2 IN MAN" CLINICAL SCIENCE, vol. 87, no. 4, 1994, pages 447-451, XP002081422 see the whole document ---	
X	C.T. CARILLI ET AL.: "HYPOTHALAMIC FACTOR INHIBITS (NA,K)ATPase FROM THE EXTRACELLULAR SURFACE" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 260, no. 2, 1985, pages 1027-1031, XP002081423 see the whole document ---	7

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10889

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
A	TYMIAK A A ET AL: "PHSICOCHEMICAL CHARACTERIZATION OF A OUABAIN ISOMER ISOLATED FROM BOVINE HYPOTHALAMUS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 17, 1 September 1993, pages 8189-8193, XP002074993 cited in the application see the whole document	1-8

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 10889

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-8
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/10889

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0433074 A	19-06-1991	AT 114475 T	15-12-1994
		CA 2032079 A	14-06-1991
		DE 69014569 D	12-01-1995
		DE 69014569 T	13-07-1995
		DK 433074 T	08-05-1995
		ES 2069030 T	01-05-1995
		GR 3015201 T	31-05-1995
		JP 4054128 A	21-02-1992
		US 5716937 A	10-02-1998